ヒト卵巣細胞への膜結合性ペプチダーゼの発現に関する研究(Dissertation_全文)

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Differential Expression of Aminopeptidase-N on Human Ovarian Granulosa and Theca Cells*

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ABSTRACT. The expression of aminopeptidase-N and neutral endopeptidase in human ovarian tissue was examined using specific monoclonal antibodies for each of these peptidases and histochemical staining for enzyme activity. Aminopeptidase-N is a membrane-bound metalloprotease catalyzing the removal of N-terminal amino acids from peptides and was detected by immunofluorescence staining on theca interna cells in secondary follicles and on luteinized thecal cells in preovulatory follicles and corpora lutea. However, aminopeptidase-N was not detected on granulosa cells. Peptidase activity was also detected by histochemical staining on theca interna cells and luteinized thecal cells. Luteinized granulosa cells showed peptidase activity, despite the lack of aminopeptidase-N. Neutral endopeptidase was not detected in ovarian granulosa and thecal cells. These observations indicate that aminopeptidase-N can be a useful surface marker for thecal cells. (*J Clin Endocrinol Metab* **74:** 91–95, 1992)

OVARIAN granulosa and thecal cells surrounding the oocyte proliferate and differentiate during follicular development and achieve luteinization to form the corpus luteum after ovulation. In this way, granulosa and thecal cells support oocyte maturation (1) and secrete sex steroid hormones in a cyclic manner under the influence of many factors, such as gonadotropins and steroid hormones (2). Furthermore, various biologically active peptides, operating under an autocrine and/or paracrine mechanism, are thought to be involved in follicular development (3, 4).

We have recently found that two distinct peptidases, neutral endopeptidase and aminopeptidase-N, are expressed on the cell surface of human endometrial stromal cells (manuscript in preparation) and have shown that interleukin-1 could modulate the growth and steroidogenesis of granulosa cells (5). Since these membrane-bound peptidases have been shown to degrade several peptide hormones and cytokines (6, 7), we thought that these peptidases, by modulating various biologically active peptides, might be concerned with follicular development. Thus, we examined the expression of aminopeptidase-N and neutral endopeptidase on human granulosa and thecal cells by immunofluorescence staining using monoclonal antibodies and by histochemical staining for peptidase activity.

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Materials and Methods

Subjects

Ovarian tissues were obtained from eight women, aged 28–46 yr, who had undergone unilateral ovarian cystectomy or oophorectomy and contralateral wedge resection for the treatment of benign ovarian cysts. The wedge-resected ovarian tissues used in this study were free of macroscopic and microscopic pathological changes. All women had histories of regular menstrual cycles (28–30 days) and had ovulatory basal body temperature charts with normal luteal phase length. Informed consent for the use of ovarian tissues for experimental purposes was obtained from each woman.

Samples

Two secondary follicles on the 7th and 8th days, three preovulatory follicles on the 13th and 14th days, one postovulatory follicle on the 15th day, and two early corpora lutea on the 18th and 19th days of the menstrual cycle were obtained. The stage of the menstrual cycle was assessed on the basis of the last menstrual period. Postovulatory follicle and early corpora lutea were reevaluated according to the histological dating of corpora lutea (8).

Antibodies

An anti-CD10 antibody, Nu-N2, which recognizes neutral endopeptidase (9), and an anti-CD13 antibody, MCS-2, which recognizes aminopeptidase-N (10), were obtained from Seikagaku-Kogyo (Osaka, Japan). Another anti-CD13 antibody, MY7, was obtained from Coulter Immunology (Hialeah, FL). The anti-CD68 antibody, DAKO-CD68/KP1, which is thought to be specific for monocytes and macrophages (11), and a developing antibody, fluorescein isothiocyanate-conjugated rabbit antimouse immunoglobulin, were obtained from DAKO Japan Co. Ltd. (Kyoto, Japan).

Immunofluorescence staining

Fresh ovarian tissue was cut into small pieces, embedded in OCT compound (Tissue-Tec, Miles Scientific, Naperville, IL), snap-frozen in liquid nitrogen, and stored at −80 C. Frozen tissues were sectioned to a 6-μm thickness using a cryostat microtome (Cryocut 1800, Reichert-Jung, Heidelberg, Germany). These sections were immediately air dried and fixed in acetone at −20 C for 5 min. Anti-CD10, anti-CD13, and anti-CD68 antibodies, which were diluted with phosphate-buffered saline (PBS) containing 0.1% BSA and 0.1% NaN_3 to 1:25, 1:25, and 1:50, respectively, were added to each section and incubated for 20 min in a moist chamber at room temperature. The slides were washed three times with PBS. Then fluorescein isothiocyanate-conjugated rabbit antimouse immunoglobulin, diluted to 1:40, was added to each section and incubated for 20 min at room temperature in the dark. The slides were washed three times with PBS, mounted with glycerin-PBS (1:1, vol/vol), and examined using a fluorescence microscope (Nikon, Tokyo, Japan).

Histochemical staining

Frozen sections for histochemical staining were prepared by the method described above. Peptidase activity was examined by the method described previously, with minor modification (12). Briefly, L-leucyl-β-naphthylamide hydrochloride (5 mg; Nakarai Tesque, Inc., Kyoto, Japan) was dissolved in 100 μL *N,N*-dimethylformamide (Nakarai Tesque, Inc., Kyoto, Japan). Fast Blue RR Salt (Sigma, St. Louis, MO; 5 mg) was dissolved in 10 mL 0.1 M PBS, pH 7.0. Then, the two solutions were mixed and filtered through a 0.22-μm membrane filter just before staining. The mixed solution was added to each section, and after incubation for 40 min at 37 C, the reaction was stopped by extensive washing with PBS. Slides were mounted in glycerol-gelatin and observed under a microscope.

Results

The secondary follicle, which consists of three layers (granulosa, theca interna, and theca externa), is shown in Fig. 1. Mitotic figures were observed in the theca interna layer of the follicle. The theca interna layer was strongly stained with antiaminopeptidase-N antibody, but not with antineutral endopeptidase antibody (Fig. 1, B and C). Neither peptidase could be detected on the granulosa cells or the theca externa layer. In accordance with the results of the immunofluorescence staining, peptidase activity was demonstrated on the theca interna layer by histochemical staining, indicating the expression of aminopeptidase-N on theca interna cells (Fig. 1D).

The preovulatory follicle, 20 mm in diameter, on the 14th day of the menstrual cycle is shown in Fig. 2. Theca interna cells are remarkably luteinized (Fig. 2A). Several mitotic figures were observed in granulosa cells. Aminopeptidase-N was expressed on luteinized theca interna cells, but not on granulosa cells (Fig. 2B). The expression of CD68 antigen was observed on sparsely scattered tissue macrophages outside the granulosa cell layer (Fig. 2C). Peptidase activity was also detected by histochemical methods on those aminopeptidase-N-positive luteinized thecal cells (Fig. 2D). Neutral endopeptidase was not detected on granulosa or thecal cells (data not shown).

In the postovulatory follicle on the 15th day of the menstrual cycle, some of the granulosa cells had become luteinized, with lightly stained abundant cytoplasm and large pale-staining nuclei. Near the thecal cells, the basement membrane was destroyed, and luteinization of granulosa cells was so well advanced that it was difficult to distinguish granulosa cells from luteinized theca interna cells. Peripheral luteinized cells strongly expressed aminopeptidase-N and could be clearly distinguished from inner aminopeptidase-N-negative and less luteinized cells (Fig. 3A). Neutral endopeptidase was not detected anywhere (data not shown). Peptidase activity was noted in all luteinized cells and originated from both granulosa and thecal cells. The intensity of activity was stronger on peripheral luteinized cells (data not shown).

The corpus luteum on the 18th day of the menstrual cycle is shown in Fig. 4. Two types of lutein cells, large and small, can be recognized (Fig. 4A). The small lutein cells are observed around the peripheral portion of the large lutein cells. These small lutein cells expressed aminopeptidase-N and could be clearly distinguished from large lutein cells (Fig. 4B). Macrophages were sparsely distributed in lutein cells and adjacent stroma (Fig. 4C). Neutral endopeptidase was not detected on either type of lutein cell (data not shown). As shown by histochemistry, both large and small lutein cells had peptidase activity (Fig. 4D).

Discussion

Antigens related to human hemopoietic cell differentiation have been defined by the cluster of differentiation (CD) designation and have been recognized by a group of monoclonal antibodies (11, 13).

CD13 antigen, recognized by the monoclonal antibody raised against promyelocytic cell line cells, was identified as aminopeptidase-N (alanine aminopeptidase) by molecular cloning of cDNA encoding CD13 antigen (10). Aminopeptidase-N is a zinc-metallopeptidase anchored in the plasma membrane; it preferentially hydrolyzes natural or synthetic substrates with an N-terminal alanine residue and has been reported to hydrolyze various biologically active peptides, such as bradykinin (14) and enkephalin (15).

CD10 antigen, termed common acute lymphoblastic leukemia antigen, was identified as neutral endopeptidase or enkephalinase by molecular cloning of cDNA encoding the antigen (9). It is also a membrane-bound zinc-metallopeptidase and is involved in the degradation of the opioid peptides, glucagon, substance-P, neurotensin, oxytocin, bradykinin, atrial natriuretic hormone, and interleukin-1 (7).

Since peptidase enzymes are widely distributed in human plasma and other tissues (6), they are thought to play an important local role in the regulation of biologically active peptides. Therefore, we took advantage of

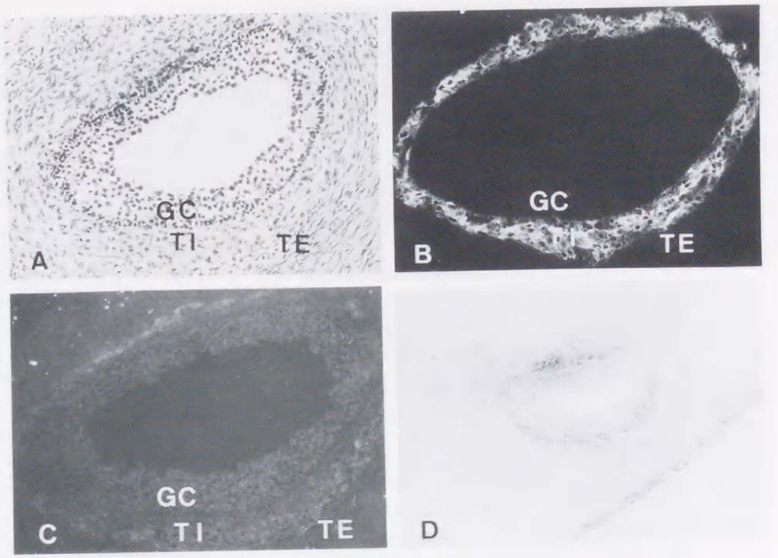

FIG. 1. Secondary growing follicle. A, Hematoxylin and eosin staining. Magnification, ×90. B, Immunofluorescence staining of aminopeptidase-N. The theca interna layer, surrounding negative granulosa cells, is strongly stained. Magnification, ×90. C, Neutral endopeptidase is negative on thecal and granulosa cells. Magnification, ×90. D, The theca interna layer is stained by histochemistry. Magnification, ×45. GC, Granulosa cells; TI, theca interna cells; TE, theca externa cells.

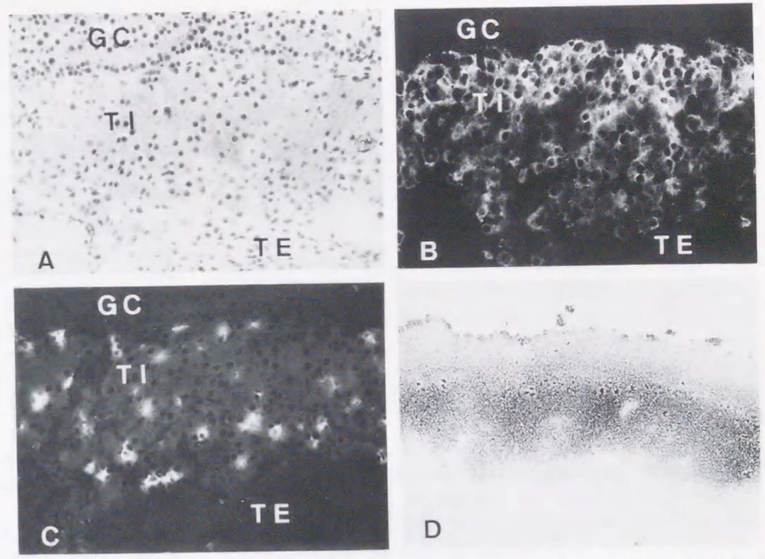

FIG. 2. Preovulatory follicle. A, Hematoxylin and eosin staining. Theca interna cells have become large and luteinized. Magnification, ×45. B, Immunofluorescence staining of aminopeptidase-N, showing clearly that luteinized theca interna cells express aminopeptidase-N, while granulosa cells do not. Magnification, ×90. C, Immunofluorescence staining of CD68 antigen. CD68 antigen-positive macrophages are distributed sparsely within the theca interna layer. Magnification, ×90. D, The luteinized theca cell layer is stained by histochemistry. Magnification, ×45. GC, Granulosa cells; TI, theca interna cells; TE, theca externa cells.

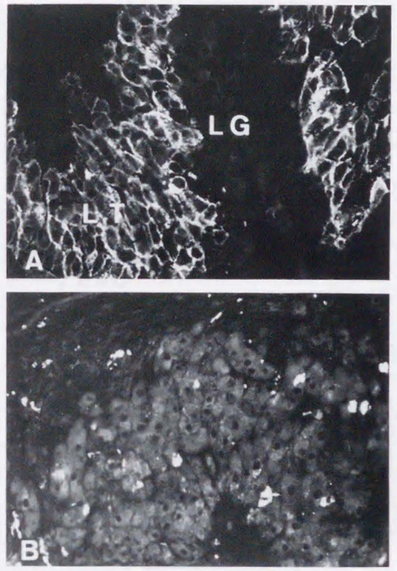

FIG. 3. Postovulatory follicle. A, Immunofluorescence staining of aminopeptidase-N. Peripheral luteinized cells, which are probably luteinized theca interna cells, as judged from their localization, strongly express aminopeptidase-N and can be clearly distinguished from inner aminopeptidase-N-negative and less luteinized cells, which are thought to be luteinizing granulosa cells. Magnification, ×90. B, Immunofluorescence staining of CD68 antigen. CD68 antigen-positive macrophages can be clearly distinguished from aminopeptidase-N-positive luteinized cells. Magnification, ×90. LG, Luteinizing granulosa cells; LT, luteinized thecal cells.

anti-CD13 and anti-CD10 antibodies to detect these peptidases in ovarian tissues in order to elucidate their possible physiological significance in the human reproductive system. In this study we detected aminopeptidase-N, but not neutral endopeptidase, on the theca interna cells of the growing follicles and on the luteinized theca interna cells of the preovulatory follicles. Aminopeptidase-N was also detected on peripheral luteinized cells, which were probably luteinized theca interna cells, as judged from their localization in the postovulatory follicle and on small lutein cells, which were thought to originate from theca interna cells (8), in the early corpora lutea. In all cases, granulosa cells expressed neither peptidase. We then thought it necessary to distinguish thecal cells expressing aminopeptidase-N from tissue macrophages that also express aminopeptidase-N. The tissue section was examined using anti-CD68 antibody, which is thought to be specific to monocytes and macrophages (11). As shown in Figs. 2C, 3B, and 4C, macrophages could be clearly distinguished from thecal cells, which were positive for aminopeptidase-N. Since theca externa cells and unluteinized ovarian stromal cells did not express it, aminopeptidase-N could be used as an excellent differentiation marker of thecal cells. It is also possible to distinguish granulosa cells from luteinized thecal cells by aminopeptidase-N expression.

Next, by histochemical staining using a synthetic substrate, L-leucyl-β-naphthylamide, we detected peptidase activity on aminopeptidase-N-positive theca interna cells and luteinized thecal cells. It should be noted that the substrate can also be hydrolyzed by some other peptidases. Interestingly, peptidase activity was detected on luteinized granulosa cells, but aminopeptidase-N could not be detected, suggesting that a peptidase other than aminopeptidase-N might exist on these cells. As far as we know, this is the first report to have demonstrated the expression of aminopeptidase-N on human endocrine cells.

Recently, a large number of biologically active peptides have been suggested to be involved in folliculogenesis, ovulation, oocyte maturation, steroid production, corpus luteum function, and luteolysis in the ovary. Furthermore, several biologically active peptides as well as steroid hormones operating under autocrine and/or paracrine mechanisms have been shown to affect the proliferation and functional differentiation of granulosa and thecal cells (3, 16, 17). Therefore, we thought it necessary to elucidate how local concentration of these biologically active peptides is regulated in the ovary.

In the endocrine system, Knights *et al.* (18) proposed that the concentration of polypeptide hormones was regulated not only by the rate of synthesis, but also by the rate of enzymatic degradation in various organs. In a neuroendocrine system on purified bovine pituitary plasma membranes, Clayton *et al.* (19) showed the existence of peptidase activity, which could inactivate GnRH; they suggested that inactivation of GnRH within the pituitary gland might be an important factor in determining the concentration of intact biologically active hormone available to the cell surface receptor site. In the nervous system some kinds of membrane-bound peptidases, including aminopeptidase-N and neutral endopeptidase, have been shown to exist on synaptic membranes and to hydrolyze several neuropeptides, suggesting that the physiological action of neuropeptides is terminated by the extracellular metabolism of these peptidases (20). Similarly, peptidases on the cell surfaces of human thecal cells can be considered to be involved in the regulation, by autocrine or paracrine mechanism, of extracellular peptide concentration.

Two kinds of regulation levels exerted by thecal cells may be proposed for the control of the local peptide concentration. As a component of target cells, the peptidase could modulate the interaction between peptides and their specific receptors just outside the plasma membrane at the cell level. As members of the follicular unit, thecal cells could affect the peptide concentration within the growing follicle or the peptide concentration of the extrafollicular stroma, including adjacent immature follicles at the tissue level.

So the expression of aminopeptidase-N on the theca interna cells in the secondary follicle suggests that membrane-bound peptidases on these endocrine cells may play an important role in folliculogenesis and oocyte maturation. Since luteinized thecal cells continue to express aminopeptidase-N after ovulation, and granulosa cells acquire peptidase activity after luteinization, peptidase might be involved in corpus luteum formation and function.

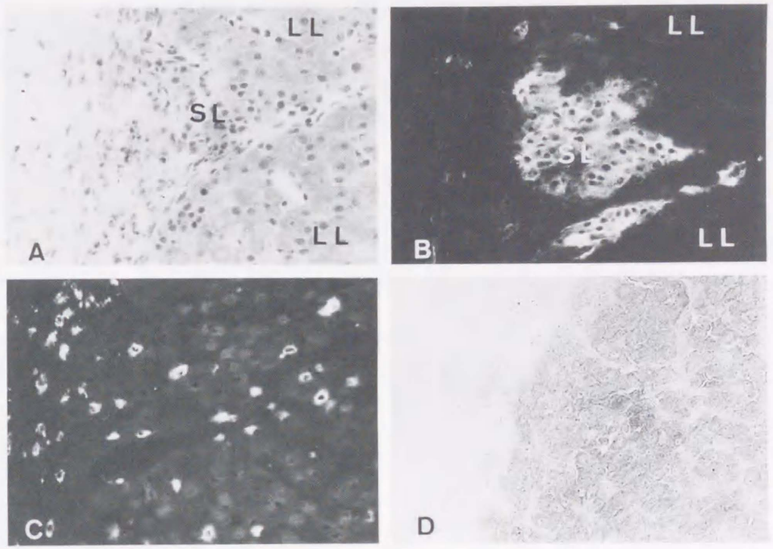

FIG. 4. Corpus luteum at an early stage (the 18th day of the menstrual cycle). A, Hematoxylin and eosin staining. Granulosa cells have become large and luteinized. Two types of lutein cells (large and small) can be recognized. Magnification, ×90. B, Immunofluorescence staining of aminopeptidase-N. Small lutein cells, which are thought to originate from thecal cells, continue to express aminopeptidase-N, surrounding the prominent large lutein cells that are negative for amiminopeptidase-N. Magnification, ×90. C, Immunofluorescence staining of CD68 antigen. CD68-positive macrophages increase and are sparsely distributed on lutein cells and surrounding stromal cells. Magnification, ×90. D, Histochemistry staining shows that both types of lutein cells have peptidase activity. Magnification, ×90. LL, Large lutein cells; SL, small lutein cells.

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Human Luteal Cells Express Dipeptidyl Peptidase IV on the Cell Surface*

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ABSTRACT

We previously reported that human theca interna cells and small luteal cells express membrane-bound aminopeptidase N, and suggested that membrane-bound peptidases are involved in folliculogenesis and luteal function by regulating extracellular peptide concentrations. In this study, we examined the expression of dipeptidyl peptidase IV (DPP IV), which is a membrane-bound peptidase and has its catalytic domain at extracellular sites, in human granulosa cells, thecal cells of growing, preovulatory, and atretic follicles, as well as corpora lutea.

Indirect immunofluorescence staining of ovarian tissues with specific monoclonal antibodies revealed that DPP IV was present in large and small luteal cells in corpora lutea. DPP IV peptidase activity was also detected histochemically in corpora lutea. In growing, preovulatory, and atretic follicles, there was weak immunoreactivity and DPP IV peptidase activity on luteinized theca interna cells, but not on granulosa cells. The expression of DPP IV on the cell surface of large and small luteal cells was confirmed by indirect immunofluorescence staining of freshly isolated luteal cells.

These results indicate that DPP IV is a useful surface differentiation marker of human luteal cells and suggest that peptidases are involved in luteal function. (*J Clin Endocrinol Metab* **75:** 1352–1357, 1992)

OVARIAN folliculogenesis is thought to be regulated mainly by gonadotropins in an endocrine manner (1). It has been recently suggested that several growth factors regulate follicular development in an autocrine and/or paracrine fashion (2). In addition, cytokines and other biologically active peptides may modulate follicular differentiation (3, 4).

Recently, we detected immunohistologically membrane-bound aminopeptidase N, which extracellularly metabolizes several biologically active peptides (5), on human theca interna cells in secondary follicles, on small luteal cells in the corpora lutea as well as on human endometrial stromal cells (6, 7). We also showed by histochemistry that these thecal cells possess aminopeptidase activity. Thus, we speculated that the membrane-bound peptidase(s) regulates the local extracellular concentrations of biologically active peptides and plays an important role in folliculogenesis. Aminopeptidase N was not detected on granulosa cells, but luteinized granulosa cells (large luteal cells) did contain peptidase activity, as revealed by histochemical staining, suggesting the presence of other peptidase(s) in large luteal cells (6).

In this study, we investigated the expression of another membrane-bound peptidase, dipeptidyl peptidase IV (DPP IV), which removes several dipeptides from the N termini of polypeptides at extracellular sites, in human ovarian follicles and corpora lutea.

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* This work was supported in part by grants-in-aid of the Ministry of Education, Science and Culture, Japan (01570927, 02454380, 02857227) and by the Shimizu Foundation Research Grant for 1990.

Subjects and Methods

Patients

Ovarian tissues were obtained from 19 women, aged 28–47 yr, who had undergone unilateral ovarian cystectomy or oophorectomy and contralateral wedge resection in the treatment of benign ovarian cysts. Wedge-resected ovarian tissues used in this study were free of macroscopic and microscopic pathological changes. All women had histories of regular menstrual cycles (28–30 days) and a normal luteal phase length on ovulatory basal body temperature charts. Informed consent for the use of ovarian tissues was obtained from each woman.

Samples

Two growing follicles on the 9th and 12th days, 2 preovulatory follicles on the 13th day and 1 on the 14th day, 3 atretic follicles on the 6th, 13th, and 20th days of the menstrual cycle, and 12 corpora lutea (CL) were obtained. The stage of the menstrual cycle was assessed on the basis of the last menstrual period. Each CL was reevaluated according to the histological dating of corpora lutea (CL day) (8). They were classified into 1 CL each at CL days 2, 4, 5, and 6, 3 CL at CL day 7, 2 CL at CL day 8, and 3 CL between CL days 10 and 12.

Antibodies

The CD26 monoclonal antibody, Ta1, which recognizes DPP IV (9, 10), was obtained from Coulter Immunology (Hialeah, FL). The CD13 monoclonal antibody, MCS-2, which recognizes aminopeptidase N (11, 12), was obtained from Seikagaku-Kogyo (Osaka, Japan) and was used for positive control staining of thecal and small luteal cells (6). The developing antibody, fluorescein isothiocyanate (FITC)-conjugated rabbit antimouse immunoglobulin, was obtained from DAKO Japan Co. LTD. (Kyoto, Japan). The negative control was the purified POG-3 monoclonal antibody of the IgG1 isotype, which specifically reacts with porcine, but not human, granulosa cells (our unpublished data).

Indirect immunofluorescence staining of ovarian tissues

Ovarian tissues were stained by the indirect immunofluorescence method as previously described (6). Briefly, fresh ovarian tissue samples were cut into small pieces, embedded in OCT compound (Tissue-Tec, Miles Scientific, Naperville, IL), snap-frozen in liquid nitrogen and stored at −80 C. Frozen tissue samples were sectioned to 7 μm thickness using a cryostat microtome (Cryocut 1800, Reichert-Jung, Heidelberg, Germany), immediately air-dried and fixed in acetone at −20 C for 5 min. CD26 (1:50) and CD13 (1:25) antibodies, diluted with phosphate buffered saline (PBS) containing 0.1% BSA and 0.1% NaN_3, were added to each section and incubated for 20 min in a moist chamber at room temperature. As a negative control, 3 μg/mL of purified POG-3 antibody was added to each section. The slides were washed three times with PBS. FITC-conjugated rabbit antimouse immunoglobulin, diluted to 1:40, was added to each section and incubated for 20 min at room temperature in the dark. The slides were washed three times with PBS, mounted with glycerin-PBS (1:1, vol/vol), and examined using a fluorescence microscope (Nikon, Tokyo, Japan).

Histochemical staining

Frozen sections for histochemical staining were prepared as described above. Peptidase activity was examined also as described previously, with minor modifications (13). Briefly, glycyl-prolyl-4-methoxy-β-naphthylamide, 4 mg (Bachem, Bubendorf, Switzerland) was dissolved in 500 μL of *N,N*-dimethylformamide (Wako Pure Chemical Industries, Ltd., Osaka). Fast Blue B Salt (Sigma Chemical Co., St. Louis, MO), 10 mg, was dissolved in 9.5 mL 0.1 M phosphate buffer, pH 7.2. The two solutions were mixed and filtered through a 0.22 μm membrane filter immediately prior to staining each section. After incubation for 10 min at room temperature, the reaction was stopped by extensive washing with PBS. The negative control was substrate-free mixed solution. Slides were mounted in glycerin-PBS (1:1, vol/vol) and observed under a microscope.

Indirect immunofluorescence staining of isolated cells from corpora lutea

For immunofluorescence staining, a single viable cell suspension was prepared from three CL at CL day 7 and one CL at CL day 8. Luteal cells were isolated as previously described, with minor modifications (14). Briefly, the CL were separated from connective tissue, then minced with scissors and incubated in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 5% fetal calf serum (Gibco, Grand Island, NY), 0.2% collagenase type I (Sigma), 0.2% hyaluronidase type I-S (Sigma), and 0.005% deoxyribonuclease I (Sigma) at 37 C for 1 h. The cell suspension was overlaid on Ficoll-Hypaque (Nacalai Tesque, Kyoto) and centrifuged for 20 min at 400 × *g*. The cells at the interphase were washed twice with Dulbecco's modified Eagle's medium and resuspended in Hanks' solution. The luteal cells, 2×10^5 were centrifuged and incubated at 4 C for 30 min with 2 μL CD26 antibody, 10 μL CD13 antibody, and 10 μL POG-3 antibody (300 μg/mL), respectively. The cells were washed with Hanks' solution twice and incubated with 20 μL FITC-conjugated rabbit antimouse immunoglobulin (diluted to 1:40) at 4 C for 30 min in the dark. The cells were then washed twice with Hanks' solution, resuspended in glycerin/PBS (1:1), mounted on glass slides, and examined using a fluorescence microscope.

Results

Indirect immunofluorescence staining of ovarian tissues

DPP IV was detected on some luteinized theca interna cells, but not on granulosa cells, of growing, preovulatory, and atretic follicles. The fluorescence intensity was, however, weak (Fig. 1A). Aminopeptidase N was highly expressed on theca interna cells in accordance with our previous report (6) (Fig. 1B).

In the CL on CL day 2 (the day after ovulation), DPP IV was detected in some peripheral luteinized cells, which are thought to originate from theca interna cells, but the intensity of the fluorescence remained weak. In the centrally located luteinizing cells, which are thought to originate from granulosa cells, DPP IV was barely detectable (Fig. 1C). Aminopeptidase N was highly expressed on the peripheral luteinized cells, but not on the centrally located luteinizing cells (6) (Fig. 1D).

In the CL on CL day 4, large luteal cells were virtually indistinguishable morphologically from small luteal cells using hematoxylin and eosin. DPP IV was detected in the peripheral luteinized cells and the fluorescence intensity appeared to have increased. In some central luteinizing cells, DPP IV was detected at very low levels (data not shown). Aminopeptidase N was highly expressed on the peripheral luteinized cells, but not on the central luteinizing cells (data not shown).

In the CL on CL days 5 and 6, large and small luteal cells were morphologically distinguishable using hematoxylin and eosin. DPP IV was expressed on small luteal cells (Fig. 2A). In some large luteal cells, DPP IV was weakly detected (Fig. 2A). Aminopeptidase N was highly expressed on small, but not large luteal cells (6) (Fig. 2B).

In the CL on CL days 7 and 8, DPP IV was expressed at higher levels on both large and small luteal cells (Fig. 2, C and D), whereas aminopeptidase N was expressed on the latter (data not shown).

In the CL on CL days 10 to 12, both large and small luteal cells continued to express DPP IV and small luteal cells also expressed aminopeptidase N (data not shown).

Histochemical staining

DPP IV peptidase activity was weak in luteinized theca interna, but it was not detected in granulosa cells of growing, preovulatory, and atretic follicles (Fig. 3A).

After ovulation, peptidase activity appeared to increase in the peripheral luteinized cells and was weakly expressed in the central luteinizing cells (Fig. 3B).

In the CL on CL days 5 and 6, both large and small luteal cells showed increased DPP IV peptidase activity, the intensity of which was stronger in small luteal cells than that in large luteal cells (data not shown).

In the CL on CL days 7 to 12, both large and small luteal cells clearly expressed DPP IV peptidase activity (Fig. 3, C and D). The peptidase activity appeared to increase more in the CL on CL days 10 to 12 than in those on CL days 7 and 8 (Fig. 3D).

Indirect immunofluorescence staining of isolated cells from corpora lutea

Large luteal cells, which were above 25 μm in diameter and contained fine lipid-like granules in the cytoplasm (Fig. 4A), expressed DPP IV on the cell surface (Fig. 4B), but not aminopeptidase N (data not shown).

Small luteal cells, which were about 20 μm in diameter and contained coarse lipid-like granules in the cytoplasm (Fig. 4, C and E), expressed both DPP IV (Fig. 4D) and aminopeptidase N (Fig. 4F) on the cell surface.

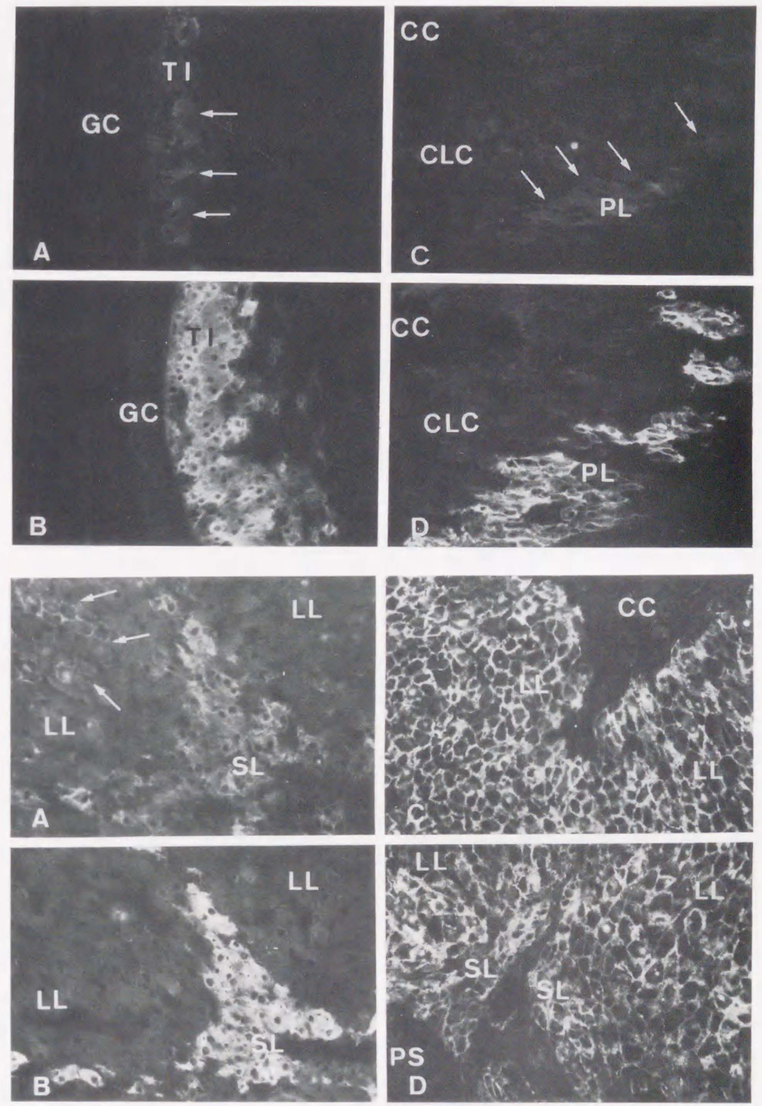

FIG. 1. The expression of DPP IV (A, C) and aminopeptidase N (B, D) in growing and postovulatory follicles detected by indirect immunofluorescence staining using specific monoclonal antibodies. Magnification, ×90. A and B, A growing follicle of 5 mm in diameter on the ninth day of the menstrual cycle. Some theca interna cells are luteinized. A, DPP IV is weakly detected in luteinized theca interna cells (*arrows*); B, aminopeptidase N is highly expressed on theca interna cells; C and D, a CL on CL day 2. C, DPP IV is expressed on some peripheral luteinized cells (*arrows*), but the fluorescence intensity is still weak. D, Aminopeptidase N is highly expressed on the peripheral luteinized cells, which are thought to originate from theca interna cells. GC, Granulosa cells; TI, theca interna cells; PL, peripheral luteinized cells; CLC, central luteinizing cells; CC, central cavity.

FIG. 2. DPP IV (A, C, and D) and aminopeptidase N (B) expression detected by immunofluorescence staining of corpora lutea using specific monoclonal antibodies. Magnification, ×90. A and B, CL on CL day 5. A, DPP IV is expressed on small luteal cells and weakly expressed on some large luteal cells (*arrows*); B, aminopeptidase N is highly expressed on small but not large luteal cells; C and D, CL on CL day 8. DPP IV is highly expressed on both large and small luteal cells. LL, large luteal cells; SL, small luteal cells; CC, central cavity; PS, peripheral stroma.

Discussion

The results of this study can be summarized as follows. 1) After ovulation, the immunoreactivity and the peptidase enzyme activity of DPP IV were detected on human luteinized granulosa cells. The intensity of DPP IV expression seemed to increase toward the mid- and late luteal phases. 2) On theca interna cells, the immunoreactivity and the peptidase enzyme activity of DPP IV seemed to increase gradually during the course of luteinization and transformation into small luteal cells. 3) DPP IV was detected on the cell surfaces of both large and small viable luteal cells isolated from corpora lutea of the midluteal phase, whereas aminopeptidase N was detected only on the cell surface of small luteal cells.

We concluded from our results that 1) DPP IV is a cell surface differentiation antigen of human granulosa cells; 2) DPP IV is a maturation or differentiation antigen of human

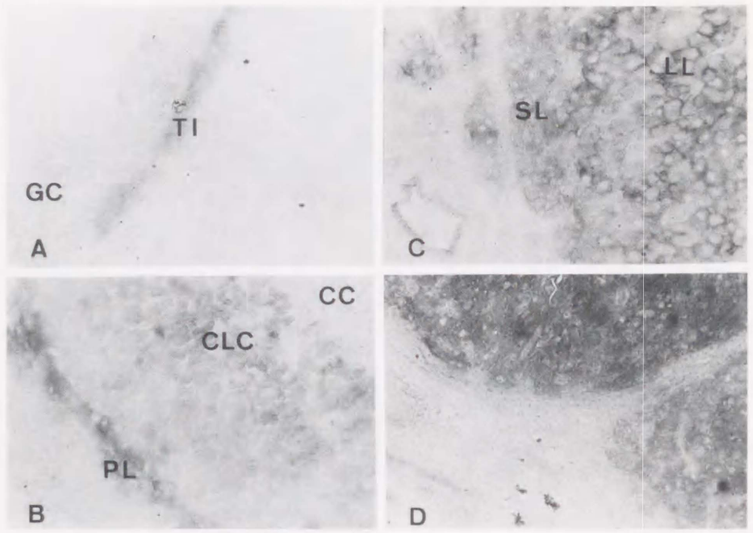

FIG. 3. DPP IV peptidase activity in ovarian follicles and corpora lutea detected by histochemical staining. Magnification, ×90. A, Preovulatory follicle on the 14th day of the menstrual cycle. The luteinized theca interna cell layer expresses DPP IV peptidase activity weakly. B, CL of CL day 4. DPP IV peptidase activity is detected on the peripheral luteinized cells and weakly on the central luteinizing cells. C, CL on CL day 7. DPP IV peptidase activity is detected on both large and small luteal cells clearly. D, CL on CL day 10. DPP IV peptidase activity is also detected on whole luteal cells. The level of expression seems to be higher than that on CL of CL day 7. GC, granulosa cells; TI, theca interna cells; CLC, central luteinizing cells; PL, peripheral luteinizing cells; CC, central cavity; LL, large luteal cells; SL, small luteal cells.

theca interna cells in the course of their luteinization and transformation into small luteal cells, when the expression of aminopeptidase N seems unchanged; 3) aminopeptidase N and DPP IV may be useful surface markers with which to distinguish small luteal cells from large luteal cells in freshly isolated cells from corpora lutea at the midluteal phase.

Previously, we raised the possibility that an unspecified peptidase(s), other than aminopeptidase N or neutral endopeptidase, is present in human luteal cells, which can hydrolyze the synthetic substrate, leucyl-β-naphthylamide. We also suggested that the peptidase(s) is involved in luteal function (6). We showed here that DPP IV, a dipeptidyl aminopeptidase, which can hydrolyze the synthetic substrate, glycyl-prolyl-4-methoxy-β-naphthylamide, is located in human luteal cells. This result supports our previous hypothesis.

DPP IV (dipeptidyl-peptide hydrolase, EC 3.4.14.5) is a membrane-bound ectoenzyme, which has an active domain at extracellular sites, and is specific for removing X amino acid (an unspecified amino acid, Xaa)-Pro dipeptides from the N termini of polypeptides and proteins. DPP IV also removes Xaa-Ala dipeptides from the N termini of polypeptides, although with 100- to 1000-fold less efficiency (15). DPP IV degrades several biologically active peptides such as substance P, GH-releasing hormone, human gastrin-releasing peptide, human pancreatic polypeptide, corticotropin-like intermediate lobe peptide, and (Tyr-)melanostatin (16, 17). The α-chain of human CG (probably including α-chains of FSH, LH, and TSH) is also a substrate for DPP IV (16). It remains to be elucidated whether this removal of dipeptides from the N-terminal of human CG is of any physiological significance for luteal cell function. However, it should be noted that removal of the Xaa-Pro dipeptide leaves these proteins unprotected against further attack by ubiquitous aminopeptidases (16) and that luteal cells appeared to express an unspecified aminopeptidase(s). A number of cytokines have DPP IV-susceptible Ala-Pro N-terminal amino acid sequences. These include interluekin-1β, interleukin-2, granulocyte-macrophage colony-stimulating factor, erythropoietin, and macrophage inflammatory protein 1a (15). The degradation of these cytokines by DPP IV has not yet been demonstrated. The finding that DPP IV is present on the cell surface of large luteal cells indicates that fully luteinized granulosa cells can metabolize biologically active peptides on the cell surface and may regulate extracellular peptide concentrations at the single cell level. This suggests that a peptide(s) is involved in luteal function.

Kenny *et al.* (18) speculated that cell-surface peptidases play a key role in the control of growth and differentiation of many cellular systems by modulating the activity of peptide factors and regulating their access to adjacent cells, although an adequate cellular system model(s) has not yet been presented. In the reproductive system, the ovary and the endometrium show dynamic changes including cellular growth, differentiation, and regression during the menstrual cycle. We have shown that three membrane-bound peptidases, namely aminopeptidase N, neutral endopeptidase, and DDP IV, are differentially expressed in these tissues. The expression of these peptidases is specific for the growth and the differentiation stages and for the different cell lineages of these tissues (6, 7). Thus, these reproductive organs can provide good models with which to investigate the hypothesis that membrane-bound peptidases are involved in cellular growth and differentiation.

DPP IV was also detected on the cell surface of activated T lymphocytes and is classified as a T lymphocyte activation-associated molecule, namely the cluster of differentiation (CD) 26 antigen (10, 19). On activated T lymphocytes, CD26 antigen is associated with an alternative pathway of signal transduction, because cross-linking of this molecule on T lymphocytes with CD26 antibody leads to further functional activation of the T lymphocytes (20). Similarly, it is possible that DPP IV on luteal cells is also involved in signal transduction.

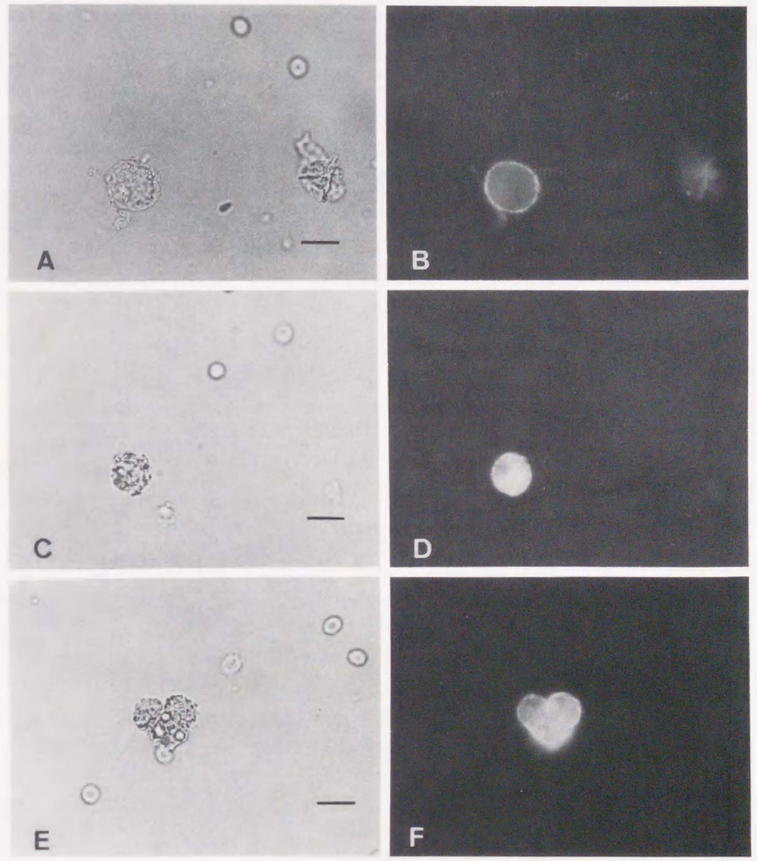

FIG. 4. Isolated luteal cells (CL day 7) showing DPP IV and aminopeptidase N on the cell surface. A, Large luteal cell, of over 25 μm in diameter, contains fine lipid-like granules in the cytoplasm; B, DPP IV is expressed on the cell surface of the large luteal cell by indirect immunofluorescence staining; C, small luteal cell, of about 20 μm in diameter, contains coarse lipid-like granules in the cytoplasm; D, DPP IV is also expressed on the cell surface of the small luteal cell by indirect immunofluorescence staining; E, three small luteal cells contain coarse lipid-like granules; F, aminopeptidase N is expressed on these small luteal cells by indirect immunofluorescence staining. The black bar indicates 22 μm.

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